Advances in the knowledge of the antiviral immune response and resistance to Viral Haemorrhagic Septicaemia Virus (VHSV) in turbot (*Scophthalmus maximus*)

DOCTORAL THESIS
Compendium of articles

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Instituto de Acuicultura
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Advances in the knowledge of the antiviral immune response and resistance to Viral Haemorrhagic Septicaemia Virus (VHSV) in turbot (*Scophthalmus maximus*)

Memoria de tesis presentada por

Patricia Pereiro González para optar al grado de Doctor por la Universidad de Santiago de Compostela

con mención internacional y en modalidad de compendio de artículos

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Dña. **BEATRIZ NOVOA GARCÍA**, Doctora en Biología y Profesora de Investigación del Consejo Superior de Investigaciones Científicas (CSIC), junto con D. **ANTONIO FIGUERAS HUERTA**, Doctor en Biología y Profesor de Investigación del Consejo Superior de Investigaciones Científico (CSIC), en calidad de directores de tesis, así como también D. **MANUEL LUIS LEMOS RAMOS**, catedrático de la Universidad de Santiago de Compostela en el departamento de Microbiología y Parasitología, en calidad de tutor,

INFORMAN:

Que la presente memoria adjunta, titulada "**Advances in the knowledge of the antiviral immune response and resistance to Viral Haemorrhagic Septicaemia Virus (VHSV) in turbot (*Scophthalmus maximus*)**", presentada por Dña. **PATRICIA PEREIRO GONZÁLEZ** para optar al grado de Doctor por la Universidad de Santiago de Compostela, con mención internacional y en modalidad de compendio de artículos, ha sido realizada bajo nuestra dirección y reúne los requisitos necesarios para ser defendida ante el tribunal calificador.

Y para que así conste, se firma la presente en Vigo, a 10 de abril de 2017.

La Doctoranda El Tutor

Fdo: Patricia Pereiro González Fdo: Dr. Manuel Luis Lemos Ramos

Los Directores de Tesis

Fdo: Dra. Beatriz Novoa García Fdo: Dr. Antonio Figueras Huerta

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Los directores de la presente tesis doctoral, presentada en formato de compendio de artículos, la Dra. **BEATRIZ NOVOA GARCÍA** y el Dr. **ANTONIO FIGUERAS HUERTA**,

DECLARAN:

Que todos los coautores de los artículos científicos incluidos en la presente tesis doctoral aceptan la presentación de los mismos como parte de la tesis doctoral presentada por Dña. PATRICIA PEREIRO GONZÁLEZ, y que todos los coautores no doctores renuncian a presentarlos en sus futuras tesis de doctorado.

Y para que así conste, se firma la presente en Vigo, a 10 de abril de 2017.

Los Directores de Tesis

Fdo: Dra. Beatriz Novoa García Fdo: Dr. Antonio Figueras Huerta

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necesitado (a pesar de que lo he "vacunado" más de una vez como si de un rodaballo más se tratase…)

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GRACIAS A TODOS, POR TODO….

A mi padre…

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RESUMO

Avances no coñecemento da resposta inmune antiviral e resistencia ao Virus da Septicemia Hemorráxica Viral (VHSV) en rodaballo (*Scophthalmus maximus*)

O rodaballo é un peixe cun alto valor comercial principalmente en Europa e na China. Aínda que o seu cultivo a día de hoxe está ben establecido, diversos patóxenos poden afectar ó seu estado sanitario, ocasionando importantes perdas económicas no sector. O virus da septicemia hemorráxica viral (VHSV) é unha das principais ameazas no cultivo do rodaballo, xa que non existen tratamentos nin vacinas comercialmente dispoñibles para este patóxeno. O obxectivo da presente tese doutoral foi, en primeiro lugar, incrementar a información dispoñible nas bases de datos no que respecta a secuencias de transcritos de rodaballos relacionadas coa resposta inmune fronte a virus. Grazas á enorme cantidade de secuencias obtidas púidose deseñar un *microarray* altamente enriquecido nestas secuencias inmunes, o que nos permitiu levar a cabo un amplo análise transcriptómico da resposta a unha infección con VHSV, así como avaliar a actividade dunha vacina de ADN fronte a VHSV tamén deseñada durante a presente tese doutoral. Esta gran cantidade de información permitiunos centrar a nosa atención en certas moléculas ou procesos que estaban a ser afectados pola vacina/infección. Este foi o caso de dous interferóns (IFNs) de tipo I, que foron caracterizados e estudados por primeira vez en rodaballo. Os IFNs de tipo I son as principais moléculas antivirais en vertebrados porque inducen a expresión de numerosos xenes capaces de bloquear a proliferación do virus. Para ampliar o coñecemento sobre este tipo de xenes tamén quixemos indagar na función do IFN de tipo II (ou IFN-gamma), o cal intervén tamén na defensa fronte aos virus pero actúa máis coma un inmunomodulador.

Palabras chave: rodaballo, VHSV, resposta inmune, interferóns de tipo I, interferón-gamma

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RESUMEN

Avances en el conocimiento de la respuesta inmune antiviral y resistencia al Virus de la Septicemia Hemorrágica Viral (VHSV) en rodaballo (*Scophthalmus maximus*)

El rodaballo (*Scophthalmus maximus*) es un pez con un alto valor comercial principalmente en Europa y China. Aunque actualmente su cultivo está bien establecido, diversos patógenos pueden afectar a su estado sanitario, ocasionando importantes pérdidas económicas en el sector. El virus de la septicemia hemorrágica viral (VHSV) es una de las principales amenazas en su cultivo, ya que no existen tratamientos ni vacunas comerciales disponibles para este patógeno. El primer objetivo de la presente tesis doctoral fue incrementar la información disponible en las bases de datos en lo que respecta a secuencias de transcritos de rodaballo relacionadas con la respuesta inmune antiviral. Gracias a la enorme cantidad de secuencias obtenidas se pudo diseñar un *microarray* altamente enriquecido en estas secuencias inmunes, lo que nos permitió llevar a cabo un amplio análisis transcriptómico de la respuesta a una infección con VHSV, así como también evaluar la actividad de una vacuna de ADN frente a VHSV diseñada durante la presente tesis doctoral. Esta gran cantidad de información nos permitió centrar nuestra atención en ciertas moléculas o procesos que estaban siendo afectados por la vacuna/infección. Este fue el caso de dos Interferones (IFNs) de tipo I, que fueron caracterizados y estudiados por primera vez en rodaballo. Los IFNs de tipo I son las principales moléculas antivirales en vertebrados porque inducen la expresión de numerosos genes capaces de bloquear la proliferación del virus. Para ampliar el conocimiento sobre este tipo de genes también quisimos indagar en la función del IFN de tipo II (o IFN-gamma), el cual interviene también en la defensa frente a virus pero actúa más como un inmunomodulador. Finalmente otro gen que llamó nuestra atención fue la Nk-lisina, por lo que lo caracterizamos y analizamos su expresión en rodaballo, encontrando una interesante correlación entre su expresión y la resistencia a VHSV.

Palabras clave: rodaballo, VHSV, respuesta inmune, interferones de tipo I, interferón-gamma

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ABSTRACT

Advances in the knowledge of the antiviral immune response and resistance to Viral Haemorrhagic Septicaemia Virus (VHSV) in turbot (*Scophthalmus maximus*)

Turbot (*Scophthalmus maximus*) is an economically valuable fish in Europe and China. Currently, the culture of this fish is well established, although several pathogens can affect its health status, causing important economic losses in the sector. Viral Haemorrhagic Septicaemia Virus (VHSV) is one of the main threats in turbot farms due to the absence of commercially available treatments and vaccines for VHSV. The first goal of this doctoral thesis was to increase the amount of information available in public databases regarding the transcriptome sequences associated with the antiviral immune response of turbot. Due to the large number of sequences obtained, a microarray highly enriched in antiviral sequences was constructed. This microarray allowed us to conduct a broad transcriptome analysis of the response to VHSV infection and evaluate the activity of a DNA vaccine against VHSV, which was also designed during this doctoral thesis. This information led us to focus our attention on certain molecules or processes affected by the vaccine/infection. This was the case for two type I interferons (IFNs), which were characterized and studied for the first time in turbot. Type I IFNs are the main antiviral molecules in vertebrates, as they induce the expression of numerous molecules with the ability to block viral proliferation. To increase our knowledge about these genes, we also sought to investigate the role of the type II IFN (or IFN-gamma), which also acts in the defence against viruses but mainly functions as an immunomodulatory molecule.

Keywords: turbot, VHSV, immune response, type I interferons, interferon-gamma

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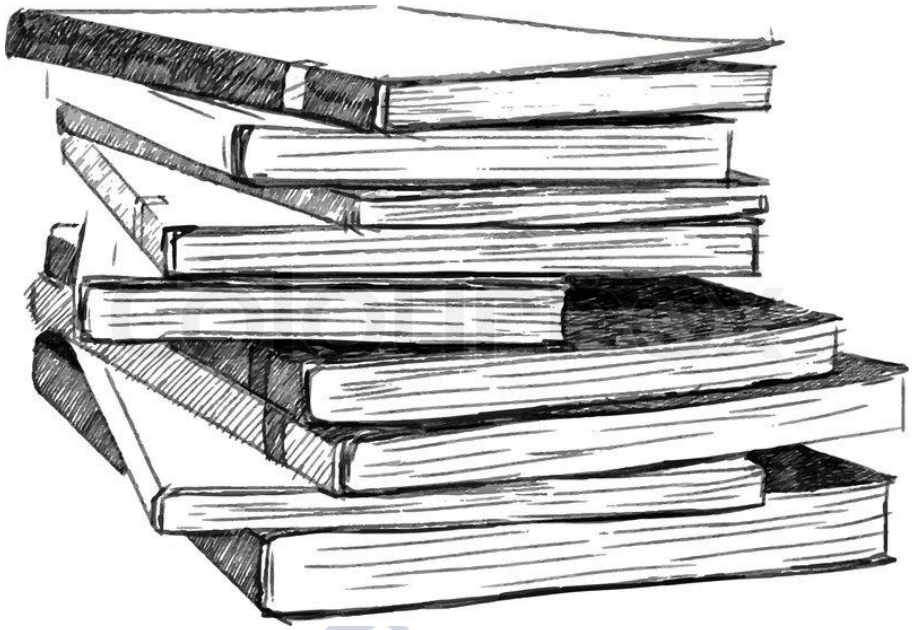

LIST OF SCIENTIFIC PUBLICATIONS AND QUALITY CRITERIA

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LIST OF PUBLICATIONS

Scientific publications included in this doctoral thesis:

Pereiro P, Balseiro P, Romero A, Dios S, Forn-Cuni G, Fuste B, Planas J V, Beltran S, Novoa B, Figueras A (**2012**) High-Throughput Sequence Analysis of Turbot (*Scophthalmus maximus*) Transcriptome Using 454-Pyrosequencing for the Discovery of Antiviral Immune Genes. PLOS ONE, 7: e35369.

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Quality criteria of the journals:

	IF 2015	5-YEAR IF	Q1	Ranking	ISSN
PLOS ONE	3.057	3.535	Multidisciplinary Sciences	11/63	1932-6203
Fish & Shellfish Immunology	3.025	3.277	Veterinary Sciences Marine and Freshwater Biology Fisheries	2/38 8/104 4/52	1050-4648
Developmental & Comparative Immunology	3.620	3.543	Zoology	6/161	0145-305X

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Forn-Cuní G, Varela M, **Pereiro P**, Novoa B, Figueras A (**2017**) Conserved gene regulation during acute inflammation between zebrafish and mammals. Scientific Reports, 7: 41905.

Book chapter:

Martínez P, Robledo D, Rodríguez-Ramilo ST, Hermida M, Taboada X, **Pereiro P**, Rubiolo JA, Ribas L, Gómez Tato A, Álvarez-Dios JA, Piferrer F, Novoa B, Figueras A, Pardo BG, Fernández J, Viñas A, Bouza C (**2016**) Turbot (*Scophthalmus maximus*) genomic resources: application for boosting aquaculture production. In: Genomics in Aquaculture, MacKenzie SA, Jentoft S (Eds.). Elsevier. pp. 131-163. ISBN: 978-0-12-801418-9

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INDEX

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

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1. GENERAL INTRODUCTION

1.1. AQUACULTURE

Following the FAO (Food and Agriculture Organization of the United Nations) definition, the term aquaculture refers to the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated, the planning, development and operation of aquaculture systems, sites, facilities and practices, and production and transport.

Currently, overfishing is an important problem mainly due to the on-going increase in the size of the human population. According to the most recent United Nations estimates, the human population of the world is expected to reach 8 billion people in 2024. Fish is a food with excellent nutritional value, providing high quality protein and a wide variety of vitamins, minerals and essential fatty acids. Globally, fish accounts for approximately 17% of animal protein intake, even exceeding 50% in many countries (Thilsted et al., 2014). Moreover, there is increasing interest, especially in developed countries, in having a healthier lifestyle, and fish represent one of the best choices for maintaining a balanced and optimal diet. For these reasons, fish consumption has increased extraordinarily during the last decades. Fortunately, aquaculture has emerged as an alternative supply of fish and shellfish. In recent years, although capture fishery production has been flat at approximately 90 million tonnes per year, aquaculture has continued to show sustained growth, amounting to 63.6 million tonnes in 2011 (Figure 1). A total of 154 million tonnes of fish were produced from all sources in 2011, of which 126 million tonnes were available for direct human consumption (Thilsted et al., 2014; FAO 2014).

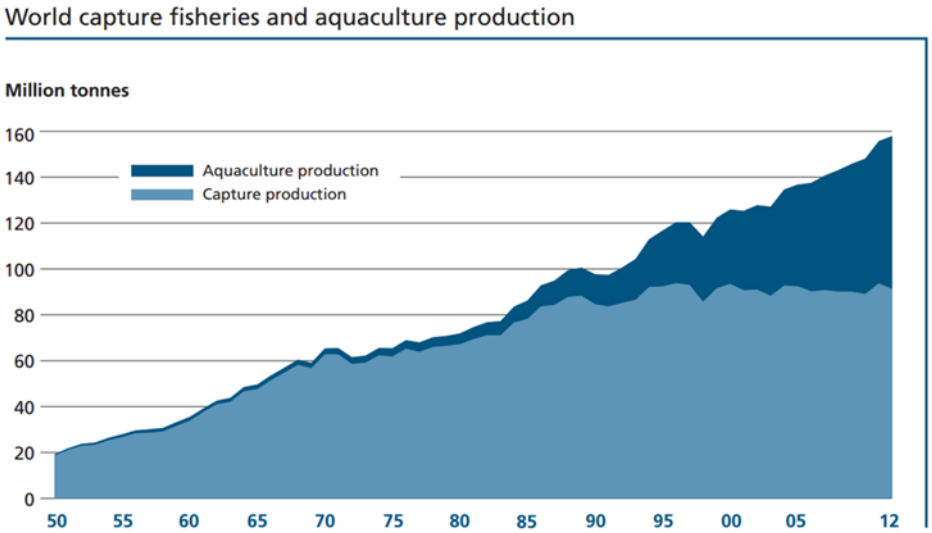

Figure 1. World capture fisheries and aquaculture production since 1950 to 2012 (FAO, 2014)

The contribution of aquaculture to global total fish production reached 43.1% in 2013, and it was only 30.6% a decade ago in 2003 (Figure 2). The FAO estimates that this percentage will reach 65% in 2030.

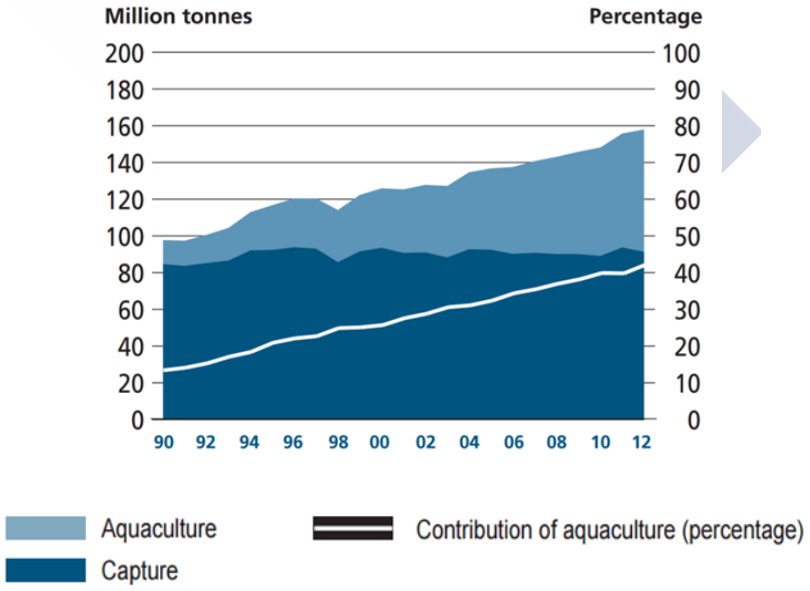

Figure 2. Share of aquaculture in total fish production (FAO, 2014)

The main producer of aquaculture products is China, followed by Indonesia, India, Vietnam, Filipinas, Bangladesh and Korea; the first European country is ranked 8th and corresponded to Norway. Fish represents almost 50% of the aquaculture in the world, although some shellfish species have very high commercial value.

In Europe, the most prominent aquaculture products are highly commercially valuable fish and molluscs. In 2012, European aquaculture accounted for 4.32% of worldwide production (excluding aquatic plants and non-food products) (FAO, 2014), but it is a leader in the culture of some species, such as Atlantic salmon, rainbow trout, sea bass, sea bream, turbot and Mediterranean mussel.

The main cultured fish species in Spain are listed in the next table (Table 1):

	YEAR									
SPECIES	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Sea Bass	5.492	8.930	10.480	9.840	13.840	12.495	14.370	14.270	14.700	17.376
Sea Bream	15.577	20.220	22.320	23.930	23.690	20.360	16.930	19.430	16.800	16.230
Portion Rainbow Trout	25.000	24.000	20.000	20.000	20.000	18.000	18.000	14.400	15.000	13.000
Turbot	4.275	5.815	6.080	7.870	8.320	6.910	7.760	7.970	6.810	7.808
Large Rainbow Trout	1.500	2.000	2.000	2.000	1.500	1.500	1.500	1.600	1.600	2.600
Meagre	273	385	810	1.300	1.660	3.250	2.880	1.640	90	1.090
Sole	60	80	60	55	180	204	110	194	313	786
European eel	405	328	360	470	510	446	402	350	315	366
Sturgeons nei	102	104	183	370	166	35	40	66	66	100
	52.684	61.862	62.293	65.835	69.866	63.200	61.992	59.920	55.694	59.356

Table 1. Spain production (tons) evolution by species (2005-2014) (FEAP, 2015)

1.2. TURBOT PRODUCTION

Turbot (*Scophthalmus maximus*) is an economically important flatfish species belonging to the family Scophthalmidae (order Pleuronectiformes) that is widely distributed from Norway to the Mediterranean and the Black Sea (Nielsen, 1986). The first steps in the production of this fish were undertaken in Scotland (United Kingdom) during the 1970s, but then turbot aquaculture was quickly expanded to Spain and France (FAO). After numerous technical and biological improvements, production was also initiated in other European countries (Portugal, Denmark, Germany, Iceland, Ireland, Italy, Norway and Wales). Currently, the culture of this fish is well established, and the complete farm-raising cycle is conducted in land-based aquaculture facilities (Figure 3). In addition to great improvements in the facilities, other decisive factors have contributed to the development of turbot aquaculture. These have included the production of dry feeds and the development of vaccines for some of the most important bacterial diseases affecting turbot (FAO).

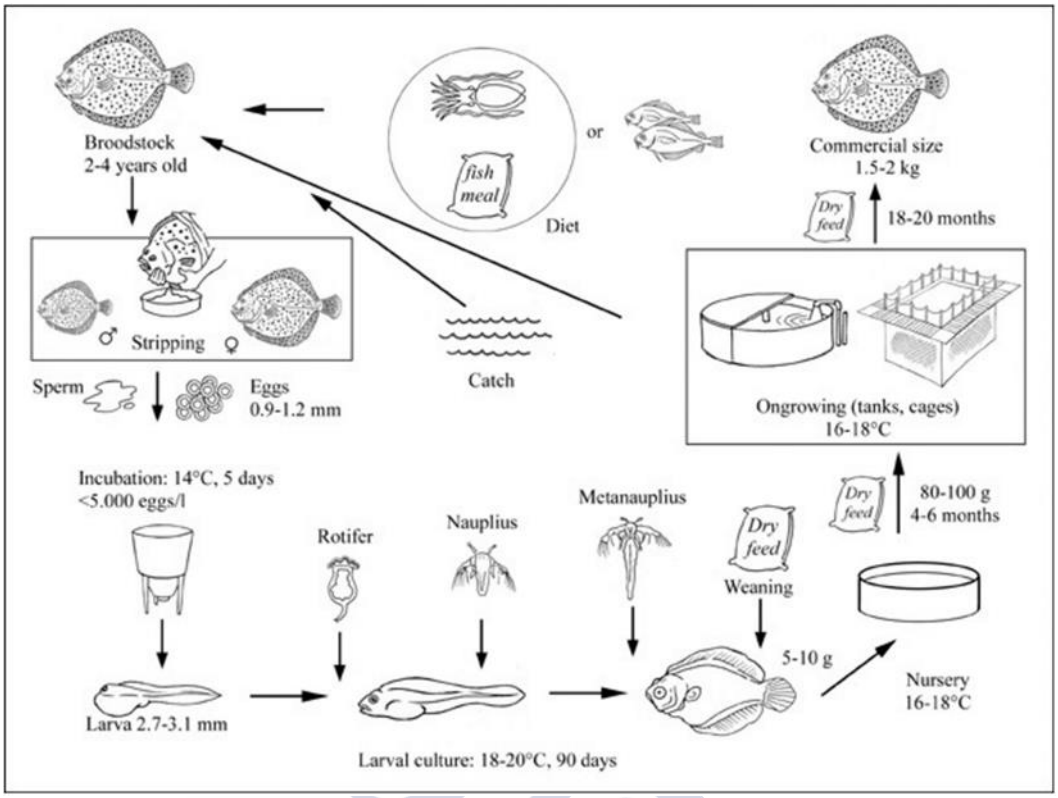

Figure 3. Production cycle of *Scophthalmus maximus* (http://www.fao.org/fishery/culturedspecies/Psetta_maxima/en)

In Europe, turbot aquaculture production was approximately 11,000 tonnes in 2014, 38.3% higher than production in 2013, with Spain (particularly the Galicia region, with 99% of national production) being the main European producer (APROMAR, 2015). Indeed, 7,808 tonnes were produced in Spain in 2014. This species, which is native to Europe, is also cultured in Chile (approximately 107 tonnes per year) but especially in China, which reached an annual level of 50,000–60,000 tonnes in recent years and is the largest producer of turbot in the world (FAO, 2010). Currently, one-third of the turbot we find in the markets in Spain comes from fisheries (APROMAR, 2015).

Nevertheless, there are currently some limitations affecting the culture of this flatfish, such as low genetic renewal and specific diseases that cause increases in mortality and morbidity, with subsequent economic losses.

1.3. DISEASES AFFECTING TURBOT CULTURE

The development of turbot aquaculture caused a parallel increase in pathological conditions affecting the culture of this flatfish. Several pathogens, including bacteria (Toranzo el at., 2005), viruses (Walker & Winton, 2010) and parasites (Álvarez-Pellitero, 2008) affect the health status of farmed fish, causing important economic losses. Despite the relevance of turbot culture and the associated pathological processes, our knowledge of its immune system is still fragmented, and little is known about host-pathogen interactions. The pathways implicated in the response against pathogens remain incomplete in fish, and understanding how these defence mechanisms act is a relevant factor in enhancing the resistance of cultured fish to diseases. Although there are currently effective treatments or vaccines available against a variety of pathogens affecting turbot culture, other diseases, especially those induced by viral agents, do not have an easy solution. Neither vaccines nor therapeutic treatments are commercially available for the most of the viral diseases affecting fish.

1.3.1. Bacterial diseases

Several bacterial pathogens can be found in turbot facilities, with four of them representing important threats to the industry. *Tenacibaculum maritimum*, the causative agent of tenacibaculosis, is a filamentous bacterium responsible for severe mortality episodes. Fortunately, a specific turbot vaccine has been developed and shows a high protection rate, but the use of antibiotics is still necessary in some cases (Avendaño-Herrera et al., 2006). The gram-positive bacterium *Streptococcus parauberis* is associated with lesions and signs of streptococcosis in cultured turbot (Domenech et al., 1996). Good protection rates were also achieved with a bacterin against this disease in turbot (Romalde et al., 1996; Toranzo et al., 1995). Two gram-negative bacteria were also implicated in disease and mortality outbreaks, *Vibrio* (*Listonella*) *anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida*. Currently, vibriosis is prevented via immersion vaccination with inactivated bacteria in small turbot (0.5-2 g). Furunculosis due to *A. salmonicida* was an extreme challenge for investigators for several years due to

dramatic mortality episodes in the salmon industry. In European turbot farms, several epizootic outbreaks of acute furunculosis have been reported (Lillehaug et al., 2003; Nougayrede et al., 1990; Pedersen et al., 1996; Toranzo & Barja, 1992). Although the application of highly effective vaccines in salmon is now a fact, a good vaccine against furunculosis is not yet commercially available for turbot. For this reason, antibiotics are needed to combat furunculosis episodes.

1.3.2. Parasitic diseases

The main parasitic agents affecting turbot culture include *Neoparamoeba pemaquidensis* (causing amoebic gill disease (AGD)), *Trichodina* spp. (trichodiniasis), *Philasterides dicentrarchi* (scuticociliatosis), *Tetramicra brevifilum* (microsporidiosis) and *Enteromyxum scophthalmi* (myxosporidiosis). The amoeba *N. pemaquidensis*, which causes severe gill tissue damage, was determined to be a causative agent of mortality in turbot cultures during the 1990s (Dyková et al., 1995, 1998). Freshwater baths are the main treatment to combat AGD. A high density of the ciliated protozoan *Trichodina* spp. can also produce skin and gill damage. It has been shown that natural infection with this parasite in cultured turbot could significantly reduce the growth rate (Sanmartín Durán et al., 1991), as was also observed in other fish species. Trichodiniasis is mainly combated using formalin baths. The first episodes of infection by histophagous scuticociliates in farmed turbot were reported in 1994 and 2000 (Dyková & Figueras 1994; Sterud et al. 2000), although it was not until 2001 that it was determined that *P. dicentrarchi* was the species responsible for these outbreaks (Iglesias et al., 2001). External signs include haemorrhagic skin ulcers and darkened skin, but when the parasite invades the internal tissues, the organs suffer important damage due to the histophagous activity of *P. dicentrarchi*. Erratic swimming, equilibrium loss, lethargy, anorexia, exophthalmia, and abdominal distension due to the accumulation of ascitic fluid in the body cavity are some of the signs observed under severe infection (Iglesias et al., 2001). Mortality can reach 100% in many cases, and it is therefore urgently necessary to develop efficient prevention and control strategies. Some laboratories are trying to find an efficient vaccine against this parasite, and some encouraging results were achieved (Palenzuela et al., 2009;

Sanmartín et al., 2008). Although *T. brevifilum* does not cause severe mortality episodes, infection by this microsporidian species can affect the growth rate and probably susceptibility to other secondary infections (Figueras et al., 1992). Finally, *E. scophthalmi* was described by Palenzuela et al. (2002) as a species causing severe catarrhal enteritis and death in cultured turbot. The mortality rate can reach up to 100%, and the absence of effective drugs against this myxosporean also represents a new challenge that needs to be solved.

1.3.3. Viral Diseases

Viruses are probably the most destructive pathogens encountered in aquaculture and are a serious concern, since no specific chemotherapies are available. Illustrating the impact of fish viruses, 8 of the 10 notifiable fish diseases (diseases with great social and economic and/or public health repercussions or present or potential risk for the aquaculture industry) appearing at the 2014 Aquatic Animal Health Code of the OIE (Office International des Epizooties, now the World Organization for Animal Health; http://www.oie.int) are caused by viruses. The most relevant viruses affecting turbot farms are recorded in this section.

Nodavirus, causing viral encephalopathy and retinopathy (VER), produces important economic losses in the larval culture of a great number of marine fish species, but only sporadic cases have been reported in turbot (Barja, 2004). In these isolated cases, turbot developed the classical signs of VER, and high mortality levels were detected (Johansen et al., 2004). Nevertheless, the susceptibility of this flatfish to nodavirus is elevated, as was demonstrated in experimental infections (Húsgağ et al., 2001; Montes et al., 2010), and therefore this disease should be taken into consideration.

IPN virus shows a similar perspective because, although it mainly causes infectious pancreatic necrosis (IPN) in salmonids, punctual cases of infection were detected in turbot (Barja, 2004). Although very different degrees of mortality were observed depending on the IPNV serotype, infected turbot do not show the typical pancreatic necrosis symptoms (Novoa et al., 1995). These investigations suggest

that turbot could act principally as an asymptomatic carrier, transferring the infection to other susceptible species.

Finally, VHSV causes an important viral disease (viral haemorrhagic septicaemia (VHS)) affecting salmonids, but VHSV outbreaks have been detected in other farmed fish species such as turbot (Ross et al., 1994; Schlotfeldt et al., 1991). The infected individuals develop the characteristic symptoms of VHS. Although the mortality rate in natural infection cases is relatively low, this rhabdovirus is included within the OIE list of notifiable diseases.

In addition to these main viral diseases, other viruses can affect turbot, although due to the lower incidence or severity in the culture of this flatfish, these are not discussed in this introduction. Some of these viruses are *Herpesvirus scophthalmi* (Hellberg et al., 2002) and erythrocytic virus (Lamas et al., 1996).

1.3.3.1 Viral Haemorrhagic Septicaemia virus (VHSV)

This aetiological agent causes an important viral disease affecting rainbow trout (*Oncorhynchus mykiss)* and other salmonids (Castric & de Kinkelin, 1980; Hørlyck et al., 1984; Wolf, 1988), but VHSV outbreaks have been detected in other farmed fish species such as turbot (Ross et al., 1994; Schlotfeldt et al., 1991). Turbot (*Scophthalmus maximus*) is a high-value farmed marine fish with growing demand and production levels in Europe and Asia. In recent years, due to intensive farming conditions, disease outbreaks caused by turbot-specific strains have frequently become severe problems faced by the turbot industry.

VHSV is a fish pathogen belonging to the genus *Novirhabdovirus* within the family *Rhabdoviridae* (Trdo et al., 2005; Walker et al., 2000). Rhabdoviruses are bullet shaped enveloped viruses 170-180 nm in length and 60-70 nm in width (Elsayed et al. 2006), with a simple negative-sense, single-stranded RNA (ssRNA) genome of approximately 11 kb (Schutze et al., 1999). The typical rhabdoviral genome encodes five basic structural proteins: nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), and large RNA-dependent RNA polymerase (L). Members of the genus *Novirhabdovirus* are distinguished by the presence of a sixth gene encoding a non-structural or non-virion (NV) protein located between the G and the L genes in the genome (Kurath

& Leong, 1985; Schutze et al., 1999); this gene has been implicated in pathogenesis (Ammayappan & Vakharia, 2011; Choi et al, 2011) (Figure 4). All rhabdoviruses possess non-coding 3' leader and 5' trailer sequences, which are also known as 3' and 5' untranslated regions (UTRs).

Figure 4. Genetic organization of the VHSV genome. The gene order of VHSV is 3'-leader-N-P-M-G-NV-L-trailer-5' (Pereiro et al., 2016)

Structurally, all rhabdoviruses have two major structural components: a helical ribonucleoprotein core (RNP) and a surrounding envelope (Figure 5). In the RNP, genomic RNA is tightly encased by the nucleoprotein. The phosphoprotein and the large protein (L-protein or polymerase) are also associated with the RNP. The glycoprotein (G) forms trimeric spikes that are tightly inserted into the lipid bilayer (typical of enveloped viruses and derived from portions of the host cell membrane). Beneath and associated with the membrane by hydrophobic and electrostatic interactions is a layer formed by the matrix protein (M), which condenses the RNP. Moreover, the M protein is also associated with the lipid bilayer and the glycoprotein, forming a link between the ribonucleocapsid and glycoproteins in the viral envelope (Assenberg et al., 2010).

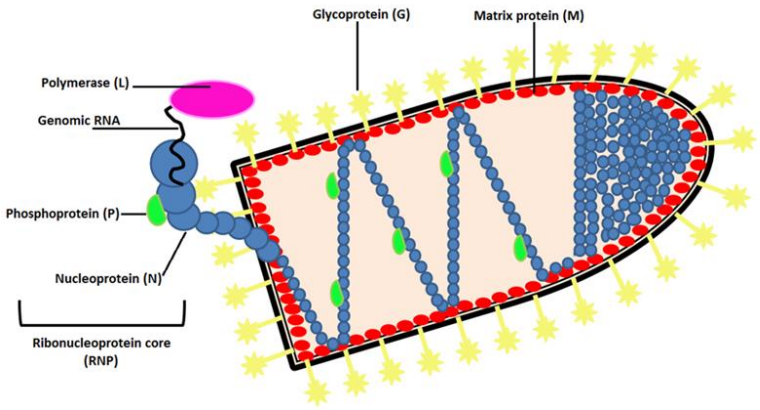

Figure 5. Schematic representation of the morphology and structural components of rhabdoviruses (Pereiro et al., 2016)

Phylogenetic analysis has allowed the identification of four major, geographically distinct VHSV genogroups based on N- and G-gene nucleotide variations (Einer-Jensen et al, 2004; Snow et al. 1999, 2004). Genotype I is composed of rainbow trout freshwater isolates (Genotype Ia) and marine isolates

from the Baltic Sea (Ib) closely related to those belonging to Ia (Snow et al., 1999). European marine strains are divided into 2 groups: Baltic Sea isolates (Genotype II) and isolates from the North Sea and European Atlantic (Genotype III). Finally, Genotype IV is composed of North American strains. In this regard, genotypes Ia and II revealed low mortality in experimentally infected turbot, while Ib showed an intermediate effect, and the highest mortality levels were obtained in turbot infected with isolates from Genotype III (Snow et al., 2005). The outbreaks detected in turbot farms were mainly caused by the UK-860/94 strain (Genotype III). Indeed, this strain was isolated from an outbreak at the Gigha turbot farm (Scotland) (Ross et al, 1994) and, although it showed low overall mortality (approximately 6%), approximately 14 tonnes of fish were consequently collected and sacrificed as a part of a contingency plan (Hastein et al., 1999), generating subsequent relevant economic losses.

Diseased fish may display nonspecific clinical signs in the early stages of infection, including the rapid onset of mortality (which can reach up to 100% in fry), lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, mouth, eyes and skin, a distended abdomen due to oedema in the peritoneal cavity, and severe abnormal swimming behaviour. Some of the symptoms we observed after the intraperitoneal injection of VHSV strain UK-860/94 in juvenile turbot are reflected in Figure 6.

Figure 6. Clinical signs in juvenile turbot infected with VHSV strain UK-860/94. External hemorrhages are observed around the eyes, mouth and fins. Internal organs also show a severe hemorrhage, especially noticeable in the liver when is compared with a healthy one (Pereiro et al., 2016).

1.3.3.2. Control and prevention of VHSV

Due to the absence of effective antiviral treatments, prevention is a critical point in the eradication of this disease. Nevertheless, no vaccines are commercially available for VHSV. For more than 30 years, increased effort has been made to produce an efficient, safe and cost-effective vaccine against VHSV using subunits or single viral proteins as well as killed or attenuated viruses (Adelmann et al, 2008; Bernard et al, 1983; de Kinkelin et al, 1980, 1995; Lecocq-Xhonneux et al, 1994; Leong & Fryer, 1993). Although some of these vaccines induced good protection levels in laboratory conditions, sometimes they are unsafe for field use, production might be very expensive or high doses may be required. Deoxyribonucleic acid (DNA) vaccination is based on the administration of a plasmid DNA vector containing the gene encoding a specific antigen. This technology is a powerful tool for the design of effective vaccines against fish pathogens. It has become clear that one of the most efficient methods for inducing a protective immune response against VHS and other Rhabdoviruses in rainbow trout under experimental conditions is DNA vaccination, with vaccines encoding viral membrane glycoproteins being remarkably efficacious (Anderson et al, 1996; LaPatra et al, 2001; Lorenzen et al, 1998, 2000; Winton, 1997). Rhabdoviruses possess a surface glycoprotein (G protein) that serves as the target of virus-neutralizing antibodies (Lorenzen et al, 1990), and the more successful DNA vaccines against these viruses are based on the G glycoprotein gene under the control of the cytomegalovirus promoter (CMV). Intramuscular administration of microgram amounts of plasmid is sufficient for the expression of the viral G glycoprotein on the surface of muscular cells, and this triggers the immune response (Lorenzen et al, 2005; Lorenzen & LaPatra, 2005).

To our knowledge, previous studies on DNA vaccination in *S. maximus* are scarce and based on protection against nodavirus infection (Sommerset et al, 2003; 2005) and the bacteria *Streptococcus iniae* (Sun et al, 2010), *Vibrio parahaemolyticus* (Liu et al, 2011) and *Vibrio harveyi* (Wang et al, 2011). During this doctoral thesis, a highly protective DNA vaccine against VHSV was developed, reflecting the potential of these vaccines in fish aquaculture.

Another way to prevent, or at least to reduce the prevalence of one disease, is genetic improvement. Marker-assisted selection (MAS) in fish breeding schemes has become a very promising strategy for obtaining individuals with a certain trait of interest. In fish aquaculture, these traits are specifically focused on growth, sex determination, and resistance to diseases. Although these markers can be morphological, biochemical or cytological, currently, most MAS work uses DNA-based markers, especially after the proliferation of genome-wide studies due to the lower cost of the genome sequencing strategies. These DNA markers can be used to detect allelic variation in the genes underlying a certain trait (Collard et al., 2005). Therefore, selection is not based on the trait itself, but on the marker linked to it. Thus, resistance to fish diseases could be improved by using DNA markers to assist in turbot breeding; this consists of the selection of allelic variations that are linked to disease resistance. The traits are usually controlled by several genes and are known as quantitative traits (Collard et al., 2005). Quantitative trait loci (QTLs) are those regions of the genome containing genes related to a quantitative trait, and the construction of physical linkage maps makes it possible to identify these chromosomal regions (Mohan et al., 1997). The marker used for selection is associated at a high frequency with the QTL of interest due to proximity on the chromosome, and therefore they should co-segregate (genetic linkage) (Mohan et al., 1997).

Numerous QTLs associated with resistance to VHSV have been identified in *S. maximus* (Rodriguez-Ramilo et al., 2014). Prior to this, QTL analyses were also used to identify those regions associated with resistance to the bacterium *Aeromonas salmonicida* (Rodríguez-Ramilo et al., 2011) and the parasite *Philasterides dicentrarchi* (Rodríguez-Ramilo et al., 2013). The existence of an accurate linkage map in turbot (Bouza et al., 2008) was crucial in the detection of these QTLs. Some QTLs were found to be related with resistance to more than one pathogen (Rodriguez-Ramilo et al., 2014), which is very interesting for designing selective breeding programmes. Until the sequencing of the turbot genome (Figueras et al., 2016), the identification of candidate genes associated with genetic markers was mainly based on comparative mapping of the turbot genetic map and the genome of model teleost species by analysing syntenic areas (Rodriguez-Ramilo et al., 2014). Currently, the whole genome sequencing of turbot has led to

the identification of numerous candidate genes associated with resistance to VHSV in a reliable and robust manner (Figueras et al., 2016). These genetic markers were located in the genome, and gene mining analysis around the QTLs was conducted using a ± 1 Mb window. Among the more than 200 candidate genes identified for VHSV resistance, some of the most remarkable findings were numerous genes implicated in T-cell activity, the blood coagulation cascade (probably due to the haemorrhagic activity of this virus) and genes related to iron homeostasis and scavenging (such as some transferrin-related genes and hepcidin) (Figueras et al., 2016) With this new information available for studies, our knowledge about the genes implicated in defence against this viral disease will probably grow in the next years.

1.4. TELEOST IMMUNE SYSTEM

1.4.1. Overview

The immune system of teleost fish is physiologically similar to that of mammals, as it consists of innate and adaptive immunity, although some differences are observed. Phylogenomic studies have suggested that, during the evolution of vertebrates, an additional genome duplication event (3R duplication) occurred in ray-finned fish (actinopterygian) 350 mya (Meyer & Van de Peer, 2005). Additionally, single gene duplication events are very common in organisms. Although most duplicated genes become non-functional (pseudogenes) after duplication, some of them evolve to acquire new functions, and the genomic complexity of the teleost is therefore generally higher than that in mammals (Meyer and Van de Peer, 2005). This is reflected in the higher number of paralog genes in fish. The existence of paralog genes in vertebrate species seems to be especially persistent in the case of immune-related genes (Flajnik & Kasahara, 2010; Ota & Nei, 1994; Piontkivska & Nei, 2003; Sarrias et al., 2004). As an example, zebrafish (*Danio rerio*) possesses four Nk-lysin genes (Pereiro et al., 2015), six perforins (Varela et al., 2016), and eight C3 complement components (Forn-Cuní, 2014). Only one copy of these genes is found in the genome of

tetrapods. Nevertheless, it is well known that paralog genes are especially abundant in zebrafish compared with other teleosts.

With the exception of lymphatic nodules and bone marrow, the remaining secondary lymphoid organs present in mammals are also found in fish (Press & Evensen, 1999). The main immune tissue is the head/anterior kidney and, together with the spleen, thymus and mucosa-associated lymphoid tissues (MALT), represents the lymphoid framework in teleosts (Press & Evensen, 1999). Moreover, fish also possess the main immune-related cell populations observed in mammals: monocytes, macrophages, granulocytes, dendritic cells, and T and B lymphocytes. The existence of natural killer cells in fish is not yet clear, although some investigations in the last decade have suggested the existence of these cells in teleost fish based on the presence of novel immune-type receptors (NITRs), the "functional orthologs" of mammalian natural killer receptors (NKRs) (Yoder, 2009).

Classically, the immune system in vertebrate organisms in divided into two main categories: innate, or non-specific, immunity and adaptive, or specific, immunity. Currently, it is known that these two systems work together to destroy microorganisms or trigger defence processes. The innate immune system is the first line of defence and can be divided into physical barriers, cellular and humoral components. Depending on the type of pathogen (bacterium, virus, parasite or fungus), and even depending on the particular species of microorganism, the immune response presents some specific components aimed at eradicating the disease. Although numerous molecules and processes are implicated in the response against viral agents, only the main components and pathways of antiviral defence in teleost fish are summarized in the next section.

1.4.2. Antiviral immune mechanisms

1.4.2.1. Virus sensors

The innate immune system's recognition of pathogens is mediated by pattern recognition receptors (PRRs). After the detection of pathogen-associated molecular patterns (PAMPs), such as bacterial and fungal glycoproteins and lipopolysaccharides or viral components, through PRRs, intracellular signalling

cascades are activated to induce the expression of numerous immune and inflammatory mediators that coordinate the elimination of pathogens and infected cells (Takeuchi & Akira, 2010).

The virus sensors, or viral PRRs, can be classified into Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain-containing (NOD)-like receptors (NLRs) (Jensen & Thomsen, 2012). Whereas TLRs are associated with the cell membrane or endosomal compartments, RLRs and NLRs patrol the cytoplasm for the presence of double-stranded viral RNA (Jensen & Thomsen, 2012) (Figure 7).

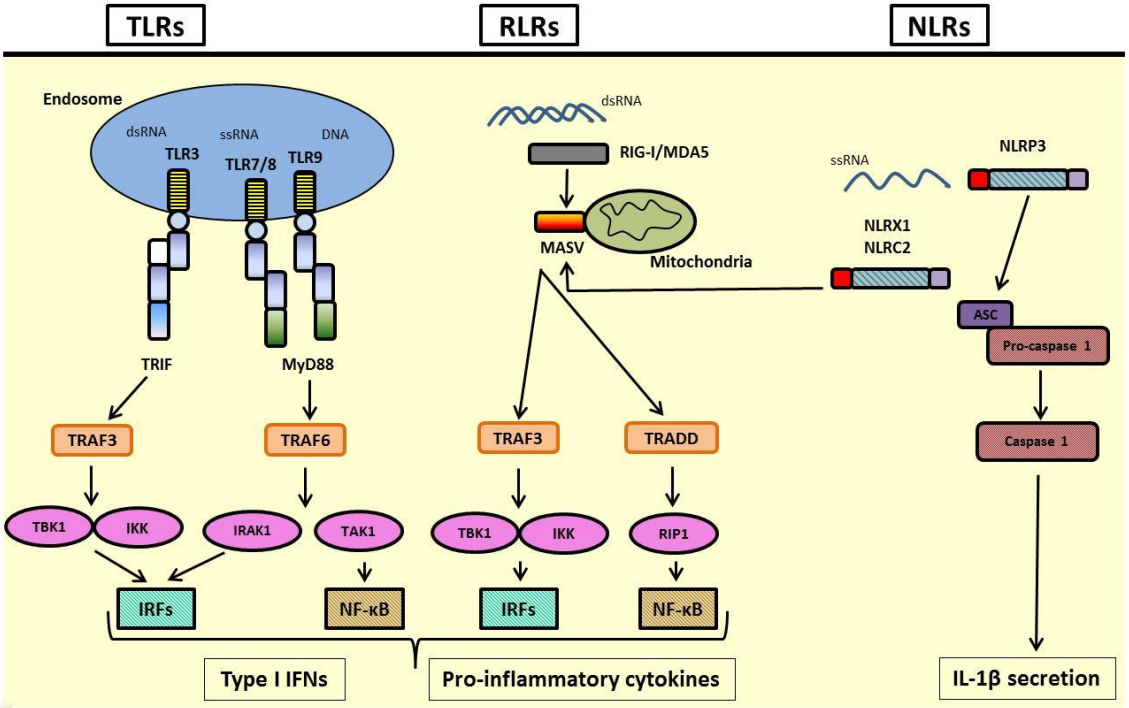

Figure 7. Viral pattern recognition receptors (PRRs).

In mammals, thirteen **TLRs** have been identified to date, of which 10 members are present in the human genome (TLR1-10) and thirteen are present in rodents (TLR1-13) (Areal et al., 2011). Each TLR recognizes specific PAMPs representing different components of pathogens (Takeda & Akira, 2004). Thus, human TLRs can be classified into non-viral (TLR1, 2, 4, 5, 6, 10) and viral TLRs (TLR3, 7, 8, 9) according to their ligand recognition (Areal et al., 2011). TLRs are composed of an ectodomain containing a variable number of leucine-rich repeats (LRRs), which is the responsible for binding PAMPs, a transmembrane segment, and a highly conserved cytoplasmic domain (TIR domain) that bind adapter

molecules (e.g., MyD88, TRIF) and triggers the intracellular cascades that finally result in the induction of numerous immune-related genes (Areal et al., 2011). The recognition of PAMPs by TLRs triggers the transcriptional up-regulation of distinct genes depending on the TLR, but mainly including type I interferons (IFNs), inflammatory cytokines and chemokines, and other molecules affecting the initiation of adaptive immune responses (Takeuchi & Akira, 2010).

In teleosts, numerous "fish-specific" TLRs have been identified in addition to those TLRs showing homology to mammals; 17 TLR types (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) were identified in more than a dozen teleost species, being in some cases duplicated in piscine genomes (Rebl et al., 2010). Orthologues of mammalian TLR6 and TLR10 were not identified in teleost species (Rebl et al., 2010), and TLR4 receptors are only present in cyprinids, although these genes do not seem to be functional receptors for bacterial lipopolysaccharide (LPS) as they are in mammals (Sullivan et al., 2009). In mammals, TLR3, 7, 8 and 9 (all of which are located on the surface of endosomes) are the main receptors responsible for virus detection; these receptors function by recognizing nucleic acids derived from viruses (Takeuchi & Akira, 2010), and this seems to be similar in fish (Rebl et al., 2010). TLR3 can detect viral replication by binding to double-stranded RNA (dsRNA), whereas TLR7 and TLR8 recognize single-stranded RNA (ssRNA), and TLR9 senses unmethylated DNA with CpG motifs (Takeuchi & Akira, 2010). In the case of teleosts, TLR22 also seems to be implicated in viral recognition by recognizing long-sized dsRNA on the cell surface (Matsuo et al., 2008). Moreover, TLR2 detects viral invasion by recognizing viral glycoproteins (Jensen and Thomsen, 2012). Until the completion of this doctoral thesis, there was only evidence for the presence of two TLRs in turbot, TLR3 (GenBank accession FJ009111) and TLR11 (Pardo et al., 2008).

The **RLRs** include three different cytoplasmic receptors: retinoic acid-inducible gene I (RIG-I, or DDX58), melanoma differentiation-associated gene 5 (MDA5, or IFIH1), and laboratory of genetics and physiology 2 (LGP2, or DHX58) (Onoguchi et al., 2011). The RLRs detect viral RNA ligands in the cytoplasm to trigger innate immunity and inflammation to control the infection, especially by activating the interferon (IFN) system (Loo & Gale, 2011). RIG-I and MDA5 possess

three domains: an N-terminal region consisting of tandem caspase activation and recruitment domains (CARDs), a central DExD/H box RNA helicase domain with the ability to hydrolyse ATP and bind to and possibly unwind RNA, and a C-terminal repressor domain (RD) embedded within the C-terminal domain (CTD) (Loo & Gale, 2011). On the other hand, LGP2 lacks the N-terminal CARDs and is currently thought to function as a regulator of RIG-I and MDA5 antiviral signalling (Rothenfusser et al., 2005; Venkataraman et al., 2007; Satoh et al., 2010; Yoneyama et al. 2004). It seems that RIG-I is preferentially activated by viral RNA bearing a triphosphate at the 5′ end and requires a short, blunt double-stranded structure for binding, whereas MDA5 has more affinity for long, double-stranded replication intermediates (Chen et al., 2015). The expression of RLRs is low in resting cells, but a great induction is generally observed after IFN exposure and viral infection (Loo & Gale, 2011). Numerous RLRs have been identified in teleost fish (Chang et al., 2011; Chen et al., 2012; Huang et al., 2010; Nie et al., 2015; Ohtani et al., 2010, 2011; Rajendran et al., 2012; Yang et al., 2011; Zou et al., 2009), although no information was available for turbot before the undertaking of this thesis. Their activity seems to be similar to that in mammals, being activated upon viral infection, polyinosine-polycytidylic acid (poly I:C) treatment and by the ubiquitin-like ISG15 protein (Langevin et al., 2013). Moreover, the downstream pathway of RLRs that includes mitochondrial antiviral signalling protein (MAVS) seems to be conserved (Biacchesi et al., 2009).

The last family of PRRs, **NLRs**, is also composed of cytosolic proteins sensing viruses. These proteins contain an LRR motif at the C terminus that functions as the sensor region, a central NACHT domain mediating oligomerization and activation, and an effector-binding domain at the N terminus (most often a CARD or PYD domain) that functions in downstream signalling (Jensen & Thomsen, 2012). A number of pathways, including the nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), inflammasome, and type I IFN signalling pathways, are activated after pathogen recognition by NLRs (Lupfer & Kanneganti, 2013). NLRP3 is the NLR most extensively studied with respect to viral infections, with a clear implication for both innate and adaptive immunity, mainly through inflammasome activation (Chakrabarti et al., 2015; Rajan et al., 2011; Thomas et al., 2009; Wang et al., 2014;). Inflammasome activation is one of the most well-

studied roles of NLRs, although not all NLRs are pro-inflammatory. The inflammasome is activated after the recognition of pathogens, mainly by NLRP1, NLRP3, and NLRC4, and it connects to caspase 1 via the adaptor molecule ASC (Latz et al., 2013). ASC forms large protein specks consisting mainly of multimers of ASC dimers and attracts monomers of pro-caspase 1 to initiate its cleavage and the formation of active caspase 1 (Latz et al., 2013). Caspase 1 mediates the cleavage of pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their active forms and is able to trigger a pro-inflammatory form of cell death known as pyroptosis (Latz et al., 2013).

Other NLRs are also involved in viral infection. These include NLRC2, which was found to reduce inflammation during viral infection via autophagy (Sabbah et al., 2009), NLRX1, which has immunomodulatory properties and the ability to produce reactive oxygen species (Allen et al., 2011; Lei et al., 2012; Moore et al., 2008), and NLRC5, a hypothetical transactivator of major histocompatibility complex (MHC) class I and antigen presentation that has contradictory results about its implication in antiviral defence mechanisms (Kuenzel et al., 2010; Kumar et al., 2011; Neerincx et al., 2010)

In addition to these virus sensors, a new family of PRRs involved in DNA sensing, termed the **AIM2-like receptors** (**ALRs**), was recently described, and **other DNA sensors** have also been discovered (Keating et al., 2011). These DNA sensors are able to discriminate between self and non-self DNA. For example, the ALRs can mediate type I IFN induction (IFI16 and p204), inflammasome activation (AIM2 and IFI16) and negative regulation (p202) (Keating et al., 2011).

1.4.2.2. The interferon system

Interferons (IFNs) are a family of multifunctional cytokines that represent the first line of defence against viral infections; these cytokines are produced in response to different PAMPs via the activation of different signalling pathways (Honda et al., 2005). In mammals, three subfamilies of IFNs were established based on differences in structural and functional properties (type I, type II and type III) (González-Navajas et al., 2012; Platanias, 2005). The type I IFN subfamily comprises a broad group of typically antiviral proteins, with interferon alpha and

interferon beta being the most studied. In contrast, the type II IFN subfamily includes only one cytokine, interferon gamma, and the third type of IFNs is the interferon lambda subfamily, which is composed of three members, none of which have been identified in fish.

Type I IFNs are the main cytokines responsible for orchestrating the antiviral response in vertebrates, but, whereas these cytokines have been largely studied in mammalian species, the knowledge of these cytokines in teleosts is more recent and limited. The first reports regarding the cloning of type I IFNs in fish were published in 2003 for zebrafish (*Danio rerio*) (Altmann et al., 2003), Atlantic salmon (*Salmo salar*) (Robertsen et al., 2003) and pufferfish (*Takifugu rubripes*) (Lutfalla et al., 2003) and, to date, type I IFNs have been reported in several teleost species (revised in Zou & Secombes, 2011). Nevertheless, no sequence for a turbot IFN, complete or partial, or numerous other interferon-related genes was available in the public databases before the investigations conducted in this PhD project.

As mentioned above, viral recognition by PRRs culminates in, among other processes, the production of type I IFNs through different downstream pathways. The activation of latent transcription factors such as NF-κB and interferon regulatory factors (IRFs) via post-translational modifications, mainly phosphorylation events, leads to the recruitment of these factors to type I IFN promoters to induce the transcription of these genes (Hiscott, 2007). When IFNs are released, they activate other cells and induce an antiviral state by interacting with the corresponding receptor (interferon alpha/beta receptor in mammals) (Samuel, 2001). This interaction induces the activation of the JAK (Janus-activated kinase)/STAT (signal transducer and activator of transcription) signalling pathway and leads to the formation of the ISGF3 (IFN-stimulated gene factor 3) complex (Samuel, 2001). This complex translocates to the nucleus and binds IFN-stimulated response elements (ISREs) in DNA to initiate the transcription of those genes known as IFN-stimulated genes (ISGs) (Platanias, 2005). These ISGs (including PKR kinase, OAS synthetase and RNase L nuclease, the family of Mx protein GTPases and ISG15, among others) reduce viral replication and dissemination through different blocking mechanisms (Sadler & Williams, 2008; Samuel, 2001).

These mechanisms for controlling all steps of viral replication include inhibition of viral transcription, degradation of viral RNA, inhibition of translation, or modification of protein function (Sadler & Williams, 2008).

IFN-gamma (type II IFN) is a markedly different IFN, possessing some ability to interfere with viral infections but mainly functioning as an immunomodulator (Boehm et al., 1997; Samuel, 2001). This cytokine is produced by different immune-related cell types, although T cells and NK cells are the major sources, and it is implicated in several aspects of immunity, such as activation of macrophages, stimulation of antigen presentation, orchestration of leukocyte-endothelium interactions, controlling cell proliferation and apoptosis, among others (Schroder et al., 2004). Regarding inflammation, IFN-gamma is typically described as a pro-inflammatory protein, although this categorization does not seem to be absolute because, in some cases, protective anti-inflammatory functions were associated with this cytokine (Mühl & Pfeilschifter, 2003; Zhang, 2007).

Unlike mammals, some bony fish, especially cyprinids, have two type II interferon genes, IFN-gamma (*ifng*) and IFN-gamma related (*ifngrel*) (Chen et al., 2010; Grayfer & Belosevic, 2009; Igawa et al., 2006; Milev-Milovanovic et al., 2006; Stolte et al., 2008). This additional gene is not a clear homologue of mammalian IFN-gamma, and it is believed than it originated after the duplication of the *ifng* gene (Zou & Secombes, 2011). The inflammatory functions of teleost type II IFNs have not been fully characterized, especially in the case of those species possessing two genes. Some studies have revealed that Ifng has the ability to induce the expression of pro-inflammatory cytokines (Arts et al., 2010; Grayfer et al., 2010; Sieger et al., 2009), whereas other investigations indicated that although zebrafish Ifng lacks the powerful pro-inflammatory activity of its mammalian counterpart, it helps to potentiate the induction of antiviral and pro-inflammatory genes by type I IFNs (López-Muñoz et al., 2009). It was observed that, as occurs in mammals, fish Ifng induces the activation of phagocytic cells by increasing the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), enhancing phagocytosis, and up-regulating the expression of different immune genes in this cell type (Arts et al., 2010; Grayfer & Belosevic, 2009; Grayfer et al., 2010; Zou et al., 2005).

1.4.2.3. Inflammation

Inflammation is a key non-specific process in viral clearance. It consists of vascular, metabolic, and cellular changes triggered by harmful stimuli in healthy tissues of the body. During the earliest stages of a viral infection, cytokines are produced when innate immune defences are activated. The activation of viral PRRs results in the production of type I IFNs and inflammatory cytokines through the activation of nuclear factor κB (NFκB) (Kawai & Akira, 2006). The main pro-inflammatory cytokines are tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interferon gamma (IFN-γ), which increase the synthesis of vasoactive substances, such as platelet-activating factor, leukotrienes, prostaglandins, and nitric oxide (NO) (Dinarello, 2000). Moreover, they are inducers of endothelial adhesion molecules, which are essential for the adhesion of leukocytes to the endothelial surface prior to emigration into tissues, and induce the synthesis of chemokines (e.g., IL-8), which are cell chemoattractants that facilitate the passage of leukocytes from the circulation to the site of inflammation (Dinarello, 2000). These cells, especially neutrophils, are critically involved in the initiation and maintenance of inflammation. Neutrophils are crucial in controlling bacterial and fungal infections via phagocytosis, degranulation (mainly reactive oxygen species and antimicrobial peptides) and neutrophil extracellular traps (NETs) (Drescher & Bai, 2013; Galani & Andreakos, 2015). Although their relevance in viral diseases seems to be critical, the exact mechanisms by which neutrophils control viral infections are still under investigation, although these mechanisms likely are similar to those implicated in bacterial infections (Drescher & Bai, 2013; Galani & Andreakos, 2015). Pro-inflammatory cytokines can also promote their own production, exhibiting autocrine, paracrine, and self-propelling effects on the inflammatory process (Wojdasiewicz et al., 2014). This positive feedback loop amplifies the response and, consequently, excessive and uncontrolled inflammation is controlled through the production of anti-inflammatory molecules (such as interleukin 10 (IL-10) or transforming growth factor beta (TGF-β)), which mainly inhibit the synthesis of inflammatory cytokines to maintain homeostasis (Dinarello, 2000; Wojdasiewicz et al., 2014).

Numerous pro-inflammatory and anti-inflammatory cytokines have been identified in teleosts. There is evidence that the main immune components implicated in inflammatory responses are present in fish (Grayfer & Belosevic, 2012). Nevertheless, as mentioned in section 4.3.1, numerous immune-related genes were found to be duplicated in teleosts. Regarding to the inflammation components, the existence of two IFNγ isoforms was confirmed in cypriniformes, which differ in their ability to modulate the inflammatory response (Grayfer & Belosevic, 2012); duplications of the IL-1β gene were also found in some fish species, although only one form seems to be functional in this case (Grayfer & Belosevic, 2012). Moreover, the presence of additional novel chemokines (Alejo & Tafalla, 2011) and PRRs (Poynter et al., 2015) in teleosts could reveal additional inflammatory pathways or mechanisms.

1.4.2.4. Antiviral strategies of cytotoxic T lymphocytes and natural killer cells

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are effector lymphocytes with the ability to eliminate tumours or virus-infected cells (Trapani & Smyth, 2002), and this mechanism seems to be highly conserved in vertebrates, including teleost fish (Nakanishi et al., 2011; Somamoto et al., 2014). Nevertheless, as mentioned above, the presence of NK cells in fish is not completely clear. There are two mechanisms for inducing the apoptosis of infected cells during a viral disease: the secretory (perforin/granzyme) pathway and the non-secretory (Fas-FasL) pathway (Trapani & Smyth, 2002). It seems that both mechanisms are indispensable for the efficient control of viral infections, as was observed through the disruption of the different pathways using murine models (Kagi et al., 1994; Parra et al., 2000; Rossi et al., 1998; Shrestha et al., 2007).

- Perforin/granzyme pathway

In the first pathway, the cytotoxic granules contained in the cytoplasm of these cell types are the protagonists. CTLs recognize viral antigens presented by major histocompatibility complex class I (MHC-I) through the T-cell receptor (TCR), and this ligation induces the activation of signalling cascades that result in polarization of the Golgi apparatus and microtubule-organizing centre and the docking and release of lytic granules (Berke, 1994). On the other hand, NK cells

recognize other signals associated with aberrant or virus-infected cells (Topham & Hewitt, 2009). The granules contain a membrane-disrupting protein known as perforin and a family of serine proteases (granzymes) implicated in the induction of apoptosis in target cells (Trapani and Smyth, 2002). Additionally, another protein, granulysin (or Nk-lysin), is present together with perforin and granzymes (Peña & Krensky, 1997). Perforin is a pore-forming member of the membrane-attack complex / PRF (MACPF) protein family. The monomers bind to the target cell membrane and polymerize in the presence of calcium to form transmembrane channels ranging from 5 to 20 nm in internal diameter and cause osmotic lysis of the target cells (Liu et al., 1995; Masson & Tschopp, 1985; Tschopp & Nabholz, 1990). Moreover, perforin allows the entry of the other cytotoxic components (mainly granzymes) into the target cell to induce apoptosis (Cullen et al., 2010; Hoves et al., 2012). To date, five different granzymes have been described in humans: granzymes A, B, H, K and M (Grossman et al., 2003). Granzyme B is the most extensively studied granzyme, and its activity is mediated by the induction of caspase-dependent apoptosis (Bots & Medema, 2006). This enzyme acts in two different ways. First, it cleaves the pro-apoptotic protein Bid, and consequently, Bid translocates to the mitochondria and together with Bax and/or Bak results in the release of pro-apoptotic proteins (such as cytochrome c) and mitochondrial outer membrane permeabilization. Granzyme B can also induce cytochrome c release through the cleavage and inactivation of the anti-apoptotic Bcl-2 family member Mcl-1. Cytochrome c is pivotal in the activation of caspase-9, which activates effector caspases. Second, granzyme B can directly process several caspases, including the effector caspase-3 and initiator caspase-8 (Bots & Medema, 2006). The other granzymes have a different a *modus operandi* but, in many cases, cell death is independent of caspase activation (Bots & Medema, 2006).

Regarding human granulysin (or Nk-lysin in other vertebrates), its function in antiviral mechanisms is not clear. This antimicrobial peptide was isolated from several vertebrate species and showed a broad antibacterial spectrum (Andersson et al., 1995; Andreu et al., 1999; Lee et al., 2014; Linde et al., 2005; Stenger et al., 1998; Zhang et al., 2014) and even antifungal (Andrä & Leippe, 1999) and antiparasitic activity (Jacobs et al., 2003; Gelhaus et al., 2008). This is due to its ability to alter membrane integrity, as occurs with the other members of the

SAPLIP family (Ruysschaert et al., 1998). Nevertheless, information about the role of this peptide in the antiviral response is very scarce. Previous works have attempted to elucidate the function of Nk-lysin/granulysin in viral diseases, but the results were contradictory in many cases. Interestingly, a microarray analysis of four turbot families showing different susceptibilities to Viral Haemorrhagic Septicaemia Virus (VHSV) revealed that Nk-lysin could be associated with resistance to the virus, as it was found to be differentially overexpressed in the highly resistant families compared with those more susceptible to viral challenge (Díaz-Rosales et al., 2012).

- Fas-FasL pathway

The second mechanism involves the engagement and aggregation of target cell death receptors (Fas) by their cognate ligand (FasL) on the killer-cell membrane, resulting in the caspase-dependent apoptosis of Fas-bearing cells (Lowin et al., 1994; Nagata & Golstein, 1995; Trapani & Smyth, 2002). Activation of CTLs through TCR interaction with viral antigens induces the expression of the FasL gene. FasL expressed on the surface of the effector cells binds to Fas on the target cell and causes apoptosis by activating caspases (Nagata, 1997). Both Fas and FasL belong to the tumour necrosis factor (TNF) family, and each contains a single transmembrane domain (Nagata, 1997). The binding of FasL with Fas instigates receptor oligomerization, which engages Fas-associated death domain (FADD); FADD activates caspase-8 through self-cleavage, which activates the effector caspases, initiating the process of apoptosis (Rauf et al., 2012).

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2. OBJECTIVES OF THIS THESIS

1. Although turbot (*S. maximus*) is a very valuable fish species both in Europe and in Asia, the gene sequence information available in public databases was very scarce until the publication of the first work that forms part of this doctoral thesis. Currently, mortality and morbidity episodes due to several pathogens, especially viral diseases, represent one of the main problems affecting the culture of this flatfish. Therefore, the first objective was to increase the transcriptome information for this species using high-throughput sequencing (454 pyrosequencing - Roche) with a special enrichment in antiviral sequences, which provided a rich source of data for further studies.

2. One of the most threatening viral diseases affecting turbot aquaculture is Viral Haemorrhagic Septicaemia (VHS). Currently, neither vaccines nor therapeutic treatments are commercially available to control the effects of VHSV. DNA vaccines have proven to be highly effective in combating salmonid fish novirhabdoviruses (VHSV and IHNV). Thus, we wanted to design a DNA vaccine encoding the viral glycoprotein in order to obtain a high protection level against VHSV in turbot.

3. The third objective of this thesis was to design a microarray (based on the transcriptome information obtained in the high-throughput sequencing of the turbot transcriptome) to analyse the transcriptome profiles after administration of the DNA vaccine against VHSV and the effect of viral infection in vaccinated and non-vaccinated fish.

4. Microarray analysis provided a large amount of transcriptomic information. The overall analysis of these data revealed interesting information about the genes implicated in the defence mechanisms against viral diseases and led us to focus our attention on some specific genes to be further studied in detail. Therefore, the last objective of the present doctoral thesis was to characterize and study the main group of antiviral cytokines, the interferon (IFN) system:

4.1. Type I IFNs (*ifn1* and *ifn2*)

4.2. Type II IFNs (*ifng*)

CHAPTER 2

High-throughput sequence analysis of the turbot (*Scophthalmus maximus*) transcriptome using 454 pyrosequencing for the discovery of antiviral immune genes

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ARTICLE

Pereiro P, Balseiro P, Romero A, Dios S, Forn-Cuni G, Fuste B, Planas J V, Beltran S, Novoa B, Figueras A (2012) High-Throughput Sequence Analysis of Turbot (*Scophthalmus maximus*) Transcriptome Using 454-Pyrosequencing for the Discovery of Antiviral Immune Genes. PLoS ONE. 7(5): e35369.

https://doi.org/10.1371/journal.pone.0035369

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CHAPTER 3

Protection and antibody response induced by intramuscular DNA vaccine encoding for viral haemorrhagic septicaemia virus (VHSV) G glycoprotein in turbot (*Scophthalmus maximus*)

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ARTICLE

Pereiro P, Martinez-Lopez A, Falco A, Dios S, Figueras A, Coll JM, Novoa B, Estepa A (2012) Protection and antibody response induced by intramuscular DNA vaccine encoding for viral haemorrhagic septicaemia virus (VHSV) G glycoprotein in turbot (*Scophthalmus maximus*). Fish & Shellfish Immunology. 32 (6): 1088–1094.

http://www.sciencedirect.com/science/article/pii/S105046481200085X

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CHAPTER 4

Transcriptome profiles associated to VHSV infection or DNA vaccination in turbot (*Scophthalmus maximus*)

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DE COMPOSTELA

ARTICLE

Pereiro P, Dios S, Boltaña S, Coll JM, Estepa A, MacKenzie S, Novoa B, Figueras A (2014) Transcriptome Profiles Associated to VHSV Infection or DNA Vaccination in Turbot (*Scophthalmus maximus*). PLoS ONE, 9(8): e104509.

https://doi.org/10.1371/journal.pone.0104509

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CHAPTER 5

The first characterization of two type I interferons in turbot (*Scophthalmus maximus*) reveals their differential role, expression pattern and gene induction

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ARTICLE

Pereiro P, Costa MM, Díaz-Rosales P, Dios S, Figueras A, Novoa B (2014) The first characterization of two type I interferons in turbot (*Scophthalmus maximus*) reveals their differential role, expression pattern and gene induction. Developmental & Comparative Immunology. 45 (2):233-244.

http://doi.org/10.1016/j.dci.2014.03.006

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CHAPTER 6

Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma

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DE COMPOSTELA

ARTICLE

Pereiro P, Forn-Cuní G, Figueras A, Novoa B. (2016) Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma. Fish & Shellfish Immunology, 59: 25-35.

http://doi.org/10.1016/j.fsi.2016.10.021

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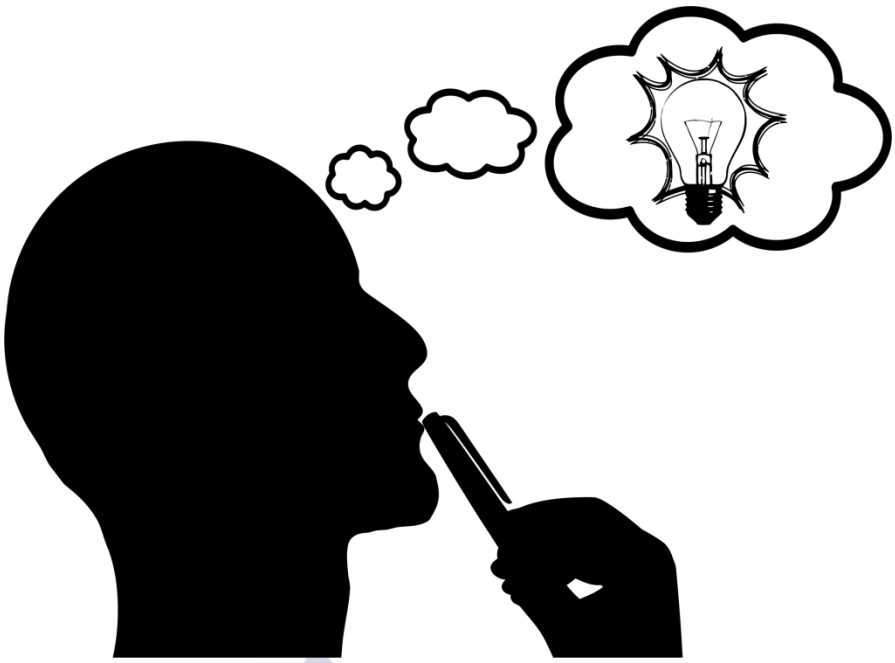

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

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1. GENERAL DISCUSSION

Turbot (*S. maximus*) is an economically important species extensively aquacultured in Europe and China. Although the production of this flatfish has undergone several improvements in recent years, one of the main threats to the sustainable growth of fish farming is infectious disease (Álvarez-Pellitero, 2008; Toranzo el at., 2005; Walker & Winton, 2010). Viral diseases are especially problematic in the finfish aquaculture industry due to the lack of antiviral therapies and difficulties in developing effective vaccines, among other questions (Dhar et al., 2014). Thus, viral disease outbreaks have caused serious economic losses all around the world. Therefore, it is not surprising that eight of the ten notifiable fish diseases appearing at the 2014 Aquatic Animal Health Code of the OIE (Office International des Epizooties, now the World Organization for Animal Health) (http://www.oie.int) are caused by viruses.

Viral Haemorrhagic Septicaemia Virus (VHSV), a virus affecting turbot production, is included on the OIE list. Knowledge about the immune response of turbot to this disease is of pivotal importance to reduce its prevalence in turbot farms. Unfortunately, until recently, the genomic or transcriptomic information available for this species in public databases was very scarce. Indeed, there were only 12,471 ESTs and fewer than 1,500 nucleotide sequences for *S. maximus* in NCBI database, most of them providing redundant information, until the publication of the first paper forming part of this thesis (**Chapter 2: High-throughput sequence analysis of the turbot (*Scophthalmus maximus*) transcriptome using 454 pyrosequencing for the discovery of antiviral immune genes**). Next-generation sequencing (NGS) technologies provide a fast and cost-effective way to generate a large amount of data from non-model species (Metzker, 2010). The aim of this work was to increase the genomic resources available for turbot, specifically the transcriptome in response to viral stimulation, and to identify the main components of the immune pathways. Our results provided a rich source of data (55,404 contigs and 181,845 singletons) for discovering and identifying new genes.

It is very important to know how turbot respond to infection with VHSV, but understanding what occurs after the vaccination process is also relevant in

identifying markers of vaccine efficacy. Due to the absence of effective antiviral treatments against VHSV, prevention is a critical point in eradication of this disease. No vaccines are commercially available for VHSV. During the last decades, several attempts were made to produce a commercially suitable vaccine against VHSV (Adelmann et al, 2008; Bernard et al, 1983; de Kinkelin et al, 1980, 1995; Lecocq-Xhonneux et al, 1994; Leong & Fryer, 1993); however, for different reasons, the transfer of these results to the industry did not occur. DNA vaccines are a powerful tool for designing effective vaccines against fish Rhabdoviruses, especially those encoding the viral membrane glycoprotein (Anderson et al, 1996; LaPatra et al, 2001; Lorenzen et al, 1998, 2000; Winton, 1997). As detailed in **Chapter 3** (**Protection and antibody response induced by an intramuscular DNA vaccine encoding viral haemorrhagic septicaemia virus (VHSV) G glycoprotein in turbot (*Scophthalmus maximus*)**), we designed a DNA vaccine encoding the G glycoprotein from VHSV strain UK-860/94, and highly promising results were obtained (relative percentage of survival (RPS) over 80%). These results reflect the potential of DNA vaccination strategies in fish aquaculture. Nevertheless, fish immunization with an antigen-encoding DNA vaccine was only approved for commercial use in Canada for IHNV (also a Novirhabdovirus) in farmed salmon (Evensen & Leong, 2013). Currently, no DNA vaccines have been approved for use in aquaculture in Europe.

As described in **Chapter 4** (**Transcriptome profiles associated to VHSV infection or DNA vaccination in turbot (*Scophthalmus maximus*)**), the transcriptome information obtained in Chapter 2 was used to construct a microarray highly enriched in antiviral sequences to analyse the transcriptome modulations in the head kidney after administration of the DNA vaccine specific for VHSV (Chapter 3) and the response to the virus in vaccinated and non-vaccinated fish. The overall analysis of these data provided interesting information about the genes implicated in the defence mechanisms against viral diseases and led us to focus our attention on some specific genes to be further characterized and studied in detail.

This was the case for type I interferons (IFNs). These cytokines induce the expression of numerous genes (interferon-stimulated genes (ISGs)) that are

growth inhibitors with the ability to reduce viral proliferation in the host through different blocking mechanisms or strategies (Sadler & Williams, 2008). For this reason, the IFN system is considered the main antiviral immune response in vertebrates. The microarray results revealed that the expression pattern of two different type I IFNs was quite different after infection with VHSV. Due to this fact and the fact that type I IFNs are the main cytokines orchestrating the antiviral immune response, **Chapter 5** (**The first characterization of two type I interferons in turbot (*Scophthalmus maximus*) reveals their differential role, expression pattern and gene induction**) describes the first characterization of two type I IFNs in turbot and analysis of their expression and bioactivity. Interestingly, these IFNs (*ifn1* and *ifn2*) showed very different activities. Ifn1 was able to induce the expression of several ISGs and, as a consequence, it induced protection against VHSV. On the other hand, Ifn2 did not induce the expression of ISGs, and it was not able to reduce viral proliferation; however, it had a function more related to immune regulation, as it was mainly involved in the inflammatory process. This is not the first time that different type I IFNs from the same fish species have shown differences in their expression patterns and protective capabilities (Aggad et al., 2009: López-Muñoz et al., 2009; Zou et al., 2007), suggesting complementary or specialized roles.

To complete the characterization of the turbot IFN repertoire, the type II IFN (or IFN-gamma) gene was also analysed in **Chapter 6** (**Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma**). Although no sequences with homology to IFN-gamma were obtained in the high-throughput sequence analysis of the turbot transcriptome described in Chapter 2, the recent publication of the turbot genome (Figueras et al., 2016) provided us with the genomic sequence of the *ifng* gene. It is well known that IFN-gamma is a markedly different IFN from type I IFNs, possessing some ability to interfere with viral infections but functioning mainly as an immunomodulatory molecule (Boehm et al., 1997; Samuel, 2001). IFN-gamma has been classically described as a pro-inflammatory cytokine, although some anti-inflammatory functions are also associated with this cytokine (Mühl & Pfeilschifter, 2003; Zhang, 2007). The most surprising result obtained in this paper was the observation that, although administration of an expression plasmid encoding turbot Ifng was not able to

reduce mortality or pathogen proliferation after viral (VHSV) or bacterial (*Aeromonas salmonicida*) challenge, this cytokine showed a dual role depending on the type of infection. It potentiated the expression of pro-inflammatory cytokines and type I IFNs during VHSV challenge, but it reduced the transcription of macrophage-related molecules. The opposite effect was observed during infection with *A. salmonicida*.

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3. CONCLUSIONS

1. The transcriptome information for turbot (*S. maximus*) was enormously enriched, especially in those sequences related to the antiviral immune response. Most of the components implicated in the main immune pathways were identified for the first time in this fish species. All this information was used to design a microarray.

2. The DNA vaccine designed during this work (pMCV1.4-G) encodes the VHSV glycoprotein (G) and was found to induce a good level of protection against VHSV. Moreover, specific anti-G neutralizing antibodies were detected in fish serum one month after vaccination, indicating activation of the adaptive immune system.

3. Microarray analysis of head kidney samples from vaccinated and non-vaccinated turbot revealed strong activation of the main immune pathways three days after vaccine administration.

4. After VHSV challenge, the transcriptome profile was completely different between vaccinated and non-vaccinated fish. Whereas naïve fish showed an extended and uncontrolled immune response, generating an intense pro-inflammatory state in the host, those individuals previously receiving the vaccine exhibited a moderate and controlled response due to the previous presence of specific immune factors.

5. Two type I IFNs (*ifn1* and *ifn2*), the main cytokines directing the antiviral immune response in vertebrates, were characterized for the first time in turbot. The results indicated non-redundant or complementary roles for these turbot IFNs. Only Ifn1 was able to induce the expression of ISGs and, as a consequence, significantly reduced the mortality of turbot upon VHSV challenge. Ifn2 seemed to act as a regulator of inflammation.

6. Turbot type II IFN (*ifng*) showed a surprising dual role depending on the type of pathogen (virus or bacteria):

a) Ifng potentiated inflammation during viral infection but had anti-inflammatory effects during bacterial disease.

b) Ifng administration had a synergistic effect on the transcription of type I IFNs during VHSV infection, but an inhibitory effect was observed when the animals were inoculated with bacteria.

c) Ifng seemed to promote the expression of those genes directly related to the activity of macrophages in *A. salmonicida*-infected turbot, but the opposite effect was observed in VHSV-infected individuals.

RESUMEN Y CONCLUSIONES EN ESPAÑOL

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Avances en el conocimiento de la respuesta inmune antiviral y resistencia al Virus de la Septicemia Hemorrágica Viral (VHSV) en rodaballo (*Scophthalmus maximus*)

1. RESUMEN

Capítulo 1: Introducción General

El rodaballo (*Scophthalmus maximus*) es un pez plano que posee un alto valor comercial especialmente en Europa y China. En la actualidad su cultivo está bien establecido, llevándose a cabo el ciclo completo principalmente en instalaciones en tierra. No obstante, existen todavía algunas limitaciones que afectan al cultivo de esta especie, como pueden ser ciertas enfermedades, las cuales causan severos episodios de mortalidad y/o morbilidad, con las subsecuentes pérdidas económicas millonarias para el sector acuícola.

El desarrollo de la acuicultura del rodaballo trajo consigo un incremento paralelo de las condiciones patológicas que afectan al cultivo de este pez. Numerosos patógenos, incluyendo bacterias, virus y parásitos pueden afectar al estado sanitario del rodaballo en mayor o menor grado. A pesar de la relevancia del cultivo de esta especie, el conocimiento sobre su sistema inmune es todavía fragmentario y poco se sabe sobre las interacciones patógeno-hospedador. Las rutas implicadas en la respuesta frente a patógenos permanecen incompletas en peces y el comprender cómo actúan estos mecanismos de defensa es un factor relevante a la hora de mejorar la resistencia a enfermedades de los peces cultivados.

Aunque a día de hoy existen tratamientos efectivos y/o vacunas disponibles frente a una gran variedad de patógenos que afectan al rodaballo, otras enfermedades, especialmente aquellas producidas por virus y endoparásitos, no tienen una solución sencilla. Los virus son probablemente los patógenos más destructivos, ya que para la mayor parte de las enfermedades virales que afectan a peces no existen vacunas ni tratamientos terapéuticos comercialmente disponibles. Para ilustrar el impacto sanitario de los virus basta con mencionar que entre las diez enfermedades de peces de declaración obligatoria que aparecen en el Código Sanitario para los Animales Acuáticos 2014 de la Organización Mundial para la Salud Animal (http://www.oie.int), ocho son causadas por virus.

Entre estas enfermedades de declaración obligatoria se encuentra la producida por el VHSV (Virus de la Septicemia Hemorrágica Viral), el cual ocasiona una importante enfermedad que afecta principalmente a la trucha arcoíris (*Oncorhyncus mykiss*) y otros salmónidos, aunque también se han detectado brotes de VHSV en otras especies de peces cultivados, como el rodaballo. Los peces que padecen la enfermedad de la septicemia hemorrágica viral presentan una serie de signos clínicos no específicos en fases tempranas de la infección, incluyendo una rápida mortalidad (la cual puede alcanzar el 100% en alevines), letargia, oscurecimiento de la piel, exoftalmia, anemia (palidez branquial), hemorragias en la base de las aletas, branquias, boca, ojos y piel, abdomen distendido debido a la acumulación de líquido ascítico y una conducta natatoria anormal. Los rodaballos infectados desarrollan los signos característicos de la enfermedad y, aunque la tasa de mortalidad debida a infecciones naturales en granjas de rodaballo es relativamente baja, esta enfermedad es de declaración obligatoria, lo que implica tomar medidas especiales en el manejo de la enfermedad que pueden agravar el impacto económico.

VHSV pertenece al género Novirhabdovirus, incluido dentro de la familia Rhabdoviridae. Se trata de virus envueltos, con un genoma de ARN de cadena sencilla el cual codifica para cinco proteínas estructurales básicas – nucleoproteína (N), fosfoproteína asociada a la polimerasa (P), proteína de matriz (M), glicoproteína (G) y ARN polimerasa dependiente del ARN (L) – y una sexta proteína no estructural, la proteína "non-virion" (NV). Existen cuatro genotipos

principales de VHSV con distinta distribución geográfica. Los brotes detectados en rodaballo son causados principalmente por la cepa UK-860/94 (Genotipo III). De hecho, esta cepa fue aislada por primera vez de un brote acontecido en una granja de rodaballo en la isla de Gigha (Escocia).

Debido a la ausencia de tratamientos antivirales efectivos la prevención es el punto crítico en la erradicación de esta enfermedad. No obstante, no existen vacunas comerciales disponibles frente a VHSV. Durante las últimas décadas se ha tratado de producir una vacuna eficaz frente a este virus usando proteínas virales así como virus muertos o atenuados y, aunque en algunos casos esas vacunas han resultado ser efectivas en condiciones experimentales, pueden no ser seguras para su uso industrial, su producción puede ser excesivamente cara o se requieren dosis muy altas. Las vacunas de ADN, basadas en la administración de un vector plasmídico que contiene un gen que codifica para un antígeno específico, han demostrado ser una herramienta altamente efectiva para el diseño de vacunas frente a rhabdovirus de peces, especialmente aquellas que codifican para la glicoproteína que constituye la membrana viral.

Otra forma de prevenir, o al menos reducir la prevalencia de una enfermedad, es la mejora genética. La selección asistida por marcadores (MAS, "Marker-assisted selection") en la cría de peces se ha convertido en una estrategia muy prometedora para obtener individuos con un cierto rasgo de interés, como puede ser la resistencia a enfermedades. En la actualidad la mayoría del trabajo en MAS usa marcadores basados en ADN, los cuales pueden ser usados para detectar variaciones alélicas en los genes que proporcionan un cierto rasgo. Estos rasgos son normalmente controlados por varios genes y las regiones del genoma que contienen los genes relacionados con estos rasgos se conocen como "quantitative trait loci" (QTLs). Los marcadores usados en la selección están asociados en una alta frecuencia con los QTLs de interés debido a la proximidad en el cromosoma, y por lo tanto son co-segregantes (ligamiento genético).

El sistema inmune de los peces teleósteos es fisiológicamente parecido al de los mamíferos, ya que poseen tanto inmunidad innata como adaptativa, aunque existen algunas diferencias. El principal órgano inmune en peces es el riñón anterior que, junto con el bazo, timo y el tejido linfoide asociado a mucosas,

representa la infraestructura linfoide en teleósteos. Además, los peces también poseen las principales poblaciones celulares presentes en mamíferos: monocitos, granulocitos, células dendríticas, y linfocitos T y B. En consecuencia, el grueso de la respuesta inmune antiviral en peces parece ser similar a la de los demás vertebrados.

El objetivo de la presente tesis doctoral fue profundizar en la respuesta frente a virus, especialmente el VHSV, en rodaballo. Dado que la principal limitación que existe a la hora de estudiar el mecanismo de defensa frente a patógenos en esta especie es la ausencia de secuencias genómicas o transcriptómicas en las bases de datos públicas, el primer objetivo fue incrementar la información transcriptómica en rodaballo, con especial enriquecimiento en secuencias que codifican para genes antivirales. Esta gran cantidad de información fue empleada en el diseño de un *microarray*, el cual nos permitió estudiar los principales rasgos de la respuesta frente a VHSV en rodaballo, así como también analizar el perfil transcriptómico tras la administración de una vacuna de ADN frente a este virus, diseñada también durante la realización de esta tesis, y comparar la respuesta diferencial al virus entre individuos vacunados y no vacunados. Este análisis global nos proporcionó valiosa información sobre la inmunidad frente a virus y nos permitió centrar nuestra atención en algunos genes específicos, los cuales fueron estudiados en más detalle. Este fue el caso de los interferones (IFNs) de tipo I, el principal grupo de citoquinas antivirales de los vertebrados, ya que los dos IFNs de tipo I presentes en el *microarray* respondían de forma diferencial frente a la infección con VHSV. Por ello fueron caracterizados y analizados de una forma más detallada con el fin de elucidar su papel concreto frente a la infección. Finalmente, tras la publicación del genoma del rodaballo en el año 2016, pudimos conocer la secuencia del interferón de tipo II (o IFN-gamma), lo que nos llevó a fijar como último objetivo de esta tesis la caracterización completa del repertorio de IFNs en rodaballo.

Capítulo 2: Análisis de alto rendimiento del transcriptoma del rodaballo mediante pirosecuenciación 454 para el descubrimiento de genes inmunes antivirales

Como se ha mencionado arriba, el rodaballo es una especie con un importante valor comercial tanto en Europa como en Asia. Sin embargo, hasta la fecha existía poca información en lo que respecta a secuencias genómicas y/o transcriptómicas en las bases de datos públicas. En la actualidad uno de los principales problemas que afectan al cultivo de este pez plano son los episodios de mortalidad debidos a diversos patógenos, especialmente aquellos ocasionados por las enfermedades virales, las cuales no tienen tratamiento comercial disponible. Con el fin de identificar nuevos genes implicados en la defensa inmune, llevamos a cabo una pirosecuenciación 454 (Roche) del transcriptoma. Numerosos individuos fueron inoculados con distintos estímulos de carácter vírico con el fin de incrementar el nivel de expresión de genes relacionados con la respuesta inmune antiviral. Se tomaron muestras de distintos tejidos a distintos tiempos post-estimulación con el fin de enriquecer lo máximo posible la información deseada. Este análisis de alto rendimiento del transcriptoma nos proporcionó 915.256 lecturas ("*reads*"), que fueron ensambladas en 55.404 *contigs*, los cuales fueron sometidos a un paso de anotación. Curiosamente, el 55,16% de las proteínas deducidas no fueron anotadas al no encontrase similitud significativa (según los criterios estadísticos establecidos) con ninguna secuencia de las bases de datos usadas en el proceso de anotación. Además, sólo un 0,85% de las secuencias fueron anotadas frente a secuencias proteicas de rodaballo, lo que viene la reflejar la escasa presencia hasta el momento de secuencias de esta especie en las bases de datos. Estos resultados sugieren la identificación de una gran cantidad de nuevos genes en rodaballo, e incluso en peces en general. Un análisis más detallado de esta información nos reveló la presencia de secuencias para una gran parte de las moléculas implicadas en las principales rutas inmunes, tanto innatas como específicas (ruta del complemento, cascada de señalización de los receptores tipo *toll*, ruta de señalización activada por los receptores de los linfocitos B y T, y apoptosis).

Este estudio supuso el primer análisis transcriptómico en rodaballo usando secuenciación masiva. Antes de llevarlo a cabo había solamente 12.471 *expressed*

sequence tags (ESTs) y menos de 1.500 secuencias nucleotídicas para *S. maximus* en las bases de datos del NCBI. Nuestros resultados supusieron una rica fuente de información (55.404 *contigs* y 181.845 *singletons*) para el descubrimiento e identificación de nuevos genes, los cuales servirán como base para la construcción de un *microarray*, para la caracterización y análisis de expresión de genes puntuales, así como también para la identificación de marcadores genéticos, entre otras aplicaciones.

Capítulo 3: Inducción de protección y producción de anticuerpos por la inyección intramuscular de una vacuna de ADN que codifica para la glicoproteína G del virus de la septicemia hemorrágica vital (VHSV) en rodaballo (*Scophthalmus maximus*)

Las vacunas de ADN que codifican para la glicoproteína viral (G) han demostrado ser las más eficaces a la hora de inducir protección frente a Rhabdovirus. Con el fin de evaluar la posibilidad de controlar los brotes de VHSV por medio de una vacuna de ADN, la glicoproteína G de una cepa de VHSV aislada de una granja de rodaballo (UK-860/94) fue clonada en un plásmido de expresión (pMCV1.4) que contiene el promotor del citomegalovirus humano. Bajo nuestras condiciones experimentales, los rodaballos a los que se les administró intramuscularmente el plásmido de expresión (pMCV1.4-G860) mostraron una protección superior al 85% frente a una infección letal con VHSV. Además, se observó que al cabo de un mes, el suero de aquellos individuos que habían sido vacunados mostraba anticuerpos específicos frente a VHSV, los cuales fueron detectados mediante *enzyme-linked immunosorbent assay* (ELISA). Midiendo la actividad neutralizante del suero se vio que estos anticuerpos presentaban capacidad neutralizante. Este trabajo supuso la primera publicación que mostraba la eficacia de la inmunización genética frente a VHSV en rodaballo.

Capítulo 4: Perfiles transcriptómicos asociados a la infección con VHSV o a la vacunación con una vacuna de ADN en rodaballo

En la actualidad los mecanismos moleculares implicados en la protección frente a patógenos todavía no se comprenden en su totalidad. Con el fin de arrojar algo de luz sobre la protección conferida por la vacuna de ADN basada en la

glicoproteína G de VHSV en rodaballo, hemos construido un *microarray* altamente enriquecido en secuencias antivirales (usando los *contigs* y algunos *singletons* obtenidos en el trabajo presentado en el capítulo 2) para llevar a cabo el estudio transcriptómico asociado a la vacunación/infección. El patrón de expresión génica en riñón anterior en respuesta a la inyección intramuscular del plásmido vacío (pMCV1.4) y la vacuna de ADN (pMCV1.4-G860) con respecto a individuos no estimulados (controles inoculados con suero salino) se analizó a 8, 24 y 72 horas post-vacunación. Además, el efecto de la infección con VHSV al cabo de un mes tras la vacunación fue también estudiado en individuos vacunados y no vacunados a los mismos tiempos de muestreo. Los genes implicados en la ruta de señalización de los receptores tipo *Toll* (*Toll-like receptors*), los genes inducidos o reguladores de la ruta de los Interferones, numerosas secuencias implicadas en la apoptosis y cascadas citotóxicas, genes relacionados con la presentación de antígenos, así como también en las cascadas del complemento y la coagulación, entre otros, fueron analizados en los diferentes grupos experimentales. Los peces que recibieron la vacuna pMCV1.4-G860 mostraron unos patrones transcriptómicos muy diferentes a los observados en los individuos inyectados con el plásmido vacío tras 72 horas, y con alto grado de similitud a los detectados tras la infección con VHSV. Por otra parte, la infección con VHSV en rodaballos vacunados y no vacunados indujo una respuesta altamente diferente a nivel transcriptómico, indicando la gran relevancia de la inmunidad adquirida o específica en los peces vacunados, capaz de alterar el perfil de respuesta inmune innata típico observado en los individuos no vacunados. Este exhaustivo estudio transcriptómico sirvió pata tener una imagen global completa con el objetivo de comprender mejor las relaciones entre la inmunidad innata y adaptativa en peces tras la infección viral/vacunación. Además, proporciona interesantes pistas sobre moléculas con un uso potencial como adyuvantes de vacunas, tratamientos antivirales o marcadores para monitorizar la eficacia de las vacunas. Lo interesante de este tipo de estudios es que permiten analizar de forma simultánea miles de genes para, a posteriori, centrarse de forma más detallada en moléculas concretas. Uno de los numerosos hallazgos que llamaron nuestra atención tras el análisis de este *microarray* fue la modulación diferencial de dos interferones (IFNs) de tipo I en rodaballo, ya que mientras uno de ellos se encontraba sobreexpresado tras la infección con VHSV, la

expresión del otro estaba inhibida. Por ello, el siguiente paso fue caracterizar y estudiar en más detalle estos dos IFNs de tipo I.

Capítulo 5: La primera caracterización de dos Interferones de tipo I en rodaballo revela sus diferencias en función, patrón de expresión e inducción génica.

Los IFNs de tipo I son considerados las principales citoquinas que dirigen y coordinan la respuesta inmune antiviral en organismos vertebrados. Estas moléculas son capaces de inducir la transcripción de numerosos genes que se conocen como genes estimulados por interferón (*interferon-stimulated genes* – ISGs) los cuales, usando diferentes mecanismos de bloqueo, reducen la proliferación viral en el hospedador. Además, se ha observado un papel contradictorio de los IFNs en la protección frente a infecciones bacterianas en roedores, incrementando la supervivencia o teniendo un efecto perjudicial dependiendo de la especie de bacteria. En teleósteos se han descrito un número variable de IFNs de tipo I dentro de una misma especie con diferentes patrones de expresión, capacidades de protección, o perfiles de inducción génica, indicando en muchos casos un papel especializado o complementario de los distintos IFNs de tipo I en la inmunidad.

En este trabajo se han caracterizado por primera vez dos IFNs de tipo I (ifn1 e ifn2) en rodaballo, los cuales mostraron distintas propiedades. Su actividad fue estudiada gracias a la producción de plásmidos de expresión que codifican para estas moléculas (pMCV1.4-ifn1 y pMCV1.4-ifn2). Aunque la expresión de ambos IFNs fue inducida tras una infección con VHSV, solamente Ifn1 presentó una clara actividad antiviral (sobreexpresión de ISGs y protección frente a VHSV), mientras que Ifn2 no fue capaz de emular esta respuesta. Por otra parte, aunque ambos genes fueron también inducidos tras la infección con *Aeromonas salmonicida* subsp. *salmonicida*, ninguno de los dos IFNs presentó efecto protector frente a la bacteria. La inyección intramuscular de los plásmidos que codifican para estos IFNs indujo la expresión de numerosos genes inmunes tanto en riñón anterior como en músculo (lugar de inyección), aunque el efecto de Ifn2 se limitó principalmente al lugar de inyección y en ningún caso indujo la expresión de ISGs, como fue mencionado con anterioridad. Curiosamente, Ifn2 pero no Ifn1 indujeron

un incremento en el nivel de expresión de interleuquina-1 beta (il1b). Por lo tanto, el papel del Ifn2 podría estar más relacionado con la regulación inmune, estando principalmente involucrado en el proceso de inflamación. Así pues, ambos IFNs de rodaballo podrían actuar de forma complementaria y diferencial durante las infecciones virales. Además, otro punto a destacar es que la sobreexpresión de il1b por Ifn2 así como la de interleuquina-8 (il8) por parte de ambos IFNs es un proceso no observado en otros vertebrados, ya que ambas moléculas son inhibidas por IFNs de tipo I en mamíferos.

Capítulo 6: La función del IFN-gamma de rodaballo es dependiente del tipo de patógeno administrado.

El IFN-gamma ha sido típicamente descrito como una citoquina pro-inflamatoria que juega un importante papel en la resolución tanto de infecciones virales como bacterianas. No obstante, algunas funciones anti-inflamatorias han sido también atribuidas a esta molécula. Con el fin de completar el repertorio de IFNs de rodaballo, en este trabajo hemos caracterizado por primera vez el gen del IFN-gamma (*ifng*) en este pez plano, cuya secuencia fue obtenida gracias a la reciente publicación de su genoma. Su patrón de expresión bajo condiciones basales, tras la administración de plásmidos de expresión codificando para IFNs de tipo I y tras la infección con virus y bacteria ha sido estudiado. La inyección intramuscular de un plásmido de expresión que codifica para el Ifng de rodaballo (pMCV1.4-ifng) no fue capaz de reducir la mortalidad causada por una infección con VHSV o *Aeromonas salmonicida* subsp. *salmonicida*. Además, la inyección del plásmido de expresión no afectó a la transcripción de numerosos genes inmunes relacionados con la actividad del IFN-gamma, con la excepción del *macrophage-colony stimulating factor* (*csf1*). Curiosamente, a las 24 horas post-infección, aquellos individuos previamente inoculados con pMCV1.4-ifng e infectados con VHSV mostraron un incremento en la expresión de citoquinas pro-inflamatorias e IFNs de tipo I en comparación con aquellos peces que no recibieron el plásmido de expresión, indicando un efecto sinérgico de Ifng y VHSV en la inducción de estos genes. Por otra parte, algunos marcadores de macrófagos, como el *macrophage receptor with collagenous structure* (*marco*) fueron inhibidos por Ifng durante la infección viral. Ifng produjo el efecto totalmente opuesto en aquellos rodaballos

infectados con bacteria, en los cuales ocasionó una reducción de la transcripción de genes pro-inflamatorios y de IFNs de tipo I, pero indujo la sobreexpresión de genes relacionados con la actividad de los macrófagos. Así pues, la actividad el Ifng de rodaballo parece ser dependiente del tipo de patógeno que causa la infección, reflejándose en este caso un claro y marcado papel dual.

2. CONCLUSIONES

1. La información transcriptómica en rodaballo (*S. maximus*) fue enormemente enriquecida, especialmente en lo que respecta a aquellas secuencias relacionadas con la respuesta inmune antiviral. Muchos de los componentes implicados en las principales rutas inmunes fueron identificados por primera vez en esta especie. Toda esta información obtenida fue usada en el diseño de un *microarray*.

2. La vacuna de ADN diseñada durante este trabajo (pMCV1.4-G), que codifica la glicoproteína G de VHSV, ha demostrado que induce buenos niveles de protección frente a VHSV. Además, un mes después de la vacunación de detectaron anticuerpos específicos frente a la glicoproteína G y con capacidad neutralizante en el suero de los rodaballos vacunados, indicando la activación del sistema inmune adaptativo.

3. Mediante el uso de *microarrays*, el análisis de las muestras de riñón anterior obtenidas de rodaballos vacunados y no vacunados reveló una fuerte activación de las principales rutas inmunes a los tres días de la administración de la vacuna.

4. Tras la infección con VHSV el perfil transcriptómico observado entre peces vacunados y no vacunados fue totalmente diferente. Mientras que los individuos que no habían sido previamente inmunizados mostraron una extensa e incontrolada respuesta inmune, lo que genera un intenso estado pro-inflamatorio en el hospedador, aquellos individuos que fueron previamente vacunados exhibieron una respuesta moderada y controlada debido a la presencia previa de factores inmunes específicos.

5. Dos IFNs de tipo I (*ifn1* y *ifn2*), que son las principales citoquinas que controlan la respuesta inmune antiviral en vertebrados, fueron caracterizados por primera vez en rodaballo. Los resultados indicaron que ambos IFNs poseen papeles no redundantes y complementarios. Solo el Ifn1 fue capaz de inducir la expresión de ISGs y, como consecuencia, de reducir de forma significativa la mortalidad tras la infección con VHSV. Ifn2 mostró una actividad que parece estar más relacionada con la regulación de la inflamación.

6. El IFN de tipo II (*ifng*) de rodaballo mostró un sorprendente papel dual dependiendo del tipo de patógeno (virus o bacteria):

a) Ifng presentó un efecto potenciador de la inflamación durante una infección bacteriana.

b) La administración de Ifng tuvo un efecto sinérgico en la transcripción de los IFNs de tipo I durante una infección con VHSV, pero se observó un efecto inhibidor cuando los animales fueron inoculados con bacteria.

c) El Ifng promovió la expresión de aquellos genes directamente relacionados con la actividad de los macrófagos en los rodaballos infectados con *A. salmonicida*, pero en aquellos individuos infectados con VHSV se observó el efecto opuesto.

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